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Cross-talk of SFRP4, integrin α1β1, and Notch1 inhibits cardiac differentiation of P19CL6 cells

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ABSTRACT

Signaling pathways play an important role in cardiogenesis. Secreted frizzled-related protein 4 (SFRP4), a member of the Wnt family, contributes to adipogenesis and tumorigenesis. However, how SFRP4 participates in cardiogenesis and the detailed molecular mechanisms involved have not been elucidated. The aim of this work was to determine cross-talk between SFRP4, integrin α1β1, and Notch1 during cardiac differentiation of P19CL6 cells. Using a well-established in vitro P19CL6 cell cardiomyocyte differentiation system, we found that SFRP4 inhibited P19CL6 cell cardiac differentiation via SFRP4 overexpression or knockdown. In addition, the SFRP4 overexpression augmented Notch1 and HES1 production. Further investigation demonstrated that SFRP4 bound to integrin α1β1 to activate the focal adhesion kinase (FAK) pathway and that phosphorylated FAK Y397 (p-FAK Y397) aided Notch intracellular domain 1 (NICD1) nuclear translocation to form a p-FAK Y397–NICD1 complex that activated the *Hes1* promoter. Taken together, the cross-talk between SFRP4, integrin α1β1, and Notch1 suppresses the cardiac differentiation of P19CL6 cells.

1. Introduction

In recent years, cell therapy has rapidly advanced as a potential strategy for treating cardiovascular diseases [1]. Remarkable progress has already been made both in basic research and clinical studies of the roles stem cells play in the therapy of heart diseases [2]. Embryonic stem cells (ESCs), bone marrow stem cells (BMSCs), and induced pluripotent stem cells (iPSCs) have been used in myocardial repair therapy studies. Despite this, the related adverse effects and ethical issues have limited their clinical applications.

Involved in cardiac differentiation, the Wnt, Notch, bone morphogenetic protein (BMP), and fibroblast growth factor (FGF) signaling pathways have been investigated extensively for their roles in cardiogenesis [3]. However, the interplay between these signaling pathways remains largely unknown.

Wnt signaling regulates a group of evolutionarily conserved pathways that play a central role in a diverse set of cellular activities [3]. Wnt/β-catenin signaling is critical for vertebrate cardiac development. Early activation of Wnt/β-catenin signaling promotes cardiac differentiation, whereas late activation of Wnt/β-catenin signaling inhibits heart formation [4].

Secreted frizzled-related proteins (SFRP1–5) are a family of glycoproteins that act as antagonists to the Wnt ligands [5]. SFRPs are composed of a netrin-like (NTR) domain and a cysteine-rich domain (CRD). The CRD shares 70% homology with Wnt receptors and competes for Wnt ligands [5].

Notch signaling pathway was comprised of four transmembrane receptors (Notch 1–4) and five transmembrane ligands (Delta-like 1, 3, 4, and Jagged 1 and 2) that are expressed on the surface of many mammalian cell types [6]. Notch1 signaling initiates cardiomyocyte differentiation by directly regulating Nkx2.5 transcription [7]. Notch4 efficiently determines the cardiac fate of hemangioblasts, resulting in the generation of populations consisting of 60% cardiomyocytes [8]. The identification of mutations in several Notch signaling components, which result in heart diseases, demonstrates the clear importance of Notch signaling in cardiogenesis.

Notch and Wnt/β-catenin signaling often intersect in stem cells and progenitor cells, and mutually regulate their transcription. The molecular mechanism of this cross-talk, however, is still obscure. It appears that cross-talk between Notch and Wnt signaling may be involved in different biological processes. The expression of Notch intracellular domain 1 (NICD1), the active form of the Notch1 intracellular domain, is dependent on Wnt/β-catenin pathway activation. Moreover, Notch1 negatively contributes to Wnt/β-catenin signaling modulation. Knockdown of Notch1 with lentivirus Notch1 short hairpin RNA (N1ShRNA) upregulates the active form of β-catenin [9]. However, the interaction between SFRPs and the Notch pathway remains unclear. SFRPs interact

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with other extracellular matrix (ECM) receptors, such as integrin receptors, except for binding with Wnt ligands or Fz receptors [10]. Integrins are transmembrane receptors that act as bridges for cell–ECM connections and cell–cell interactions. Integrin receptors are obligate heterodimers composed of two different chains, termed α and β subunits. The α1, α3, α5, α7, β1, and β3 subunits are mainly expressed in cardiomyocytes [11]. The initiation of the terminal differentiation of cells can be partially inhibited by adhesion-blocking antibodies to integrin β1. Nevertheless, the role of integrin in cardiac differentiation is unclear [12].

This study first reports that SFRP4 inhibits the cardiac differentiation of P19CL6 cells by binding with integrin α1β1 to accelerate focal adhesion kinase (FAK) Y397 phosphorylation, while the activated FAK promotes NICD1 translocation into the nucleus, and FAK Y397 and NICD1 form a complex to inhibit the expression of cardiac genes. Our study uncovers the cross-talk of SFRP4, integrin α1β1, and the Notch1 pathway, which serves to increase our understanding of heart development.

2. Materials and methods

2.1. Cell culture, induction of differentiation, and reagents

P19CL6 cells were cultured as described previously [14]. Briefly, the cells were grown in a 60-mm tissue culture–grade dish under adherent conditions with minimal essential medium supplemented with 10% fetal bovine serum (FBS, HyClone), penicillin (100 U/ml), and streptomycin (100 U/ml) (growth medium), and were maintained in 5% CO_2 at 37 °C.

To induce cardiac differentiation under adherent conditions, the cells were plated at a density of 3.7×10^5 in a 60-mm tissue culture–grade dish with growth medium containing 1% dimethyl sulfoxide (DMSO, Sigma-Aldrich) (differentiation medium), which was changed every other day. The days of differentiation were numbered consecutively after the first day of DMSO treatment (day 0).

Recombinant SFRP4 (100 ng/ml; 9115G2, Sigma-Aldrich) was added to the cell cultures every 2 days.

2.2. Real-time reverse transcription (RT)-PCR

Total RNA extraction from P19CL6 cells and real-time RT-PCR were performed as previously described [13]. Transcript levels were normalized to 18S rRNA levels. The primers are listed in Table 1. Each value represents the average of at least three independent experiments.

2.3. Plasmid transfection and RNA interference

P19CL6 cells were transfected with plasmids or synthesized small interfering RNA (siRNA) for gene overexpression or knockdown, respectively. The plasmid carrying NICD1 (pCDNA3.1-NICD1) was a kind gift from Dr. Martin Baron (University of Manchester). The plasmid carrying FAK was a kind gift from Dr. Weiguo Zhu (Peking University Health Science Center). The SFRP4 plasmid was a kind gift from Dr. Arattner (Johns Hopkins University). Plasmid and siRNA transfection was performed using Lipofectamine 2000 and Lipofectamine RNAiMAX (Invitrogen), respectively, according to the manufacturer's protocols. After 48-hour transfection, the cells were harvested for functional assays. All experiments were performed at least three times.

2.4. Western blotting

Western blotting was performed as previously described [14]. The antibodies used were against SFRP4 (9115G2, Sigma-Aldrich), integrin α1 (sc-271034, Santa Cruz), integrin (ITG) β1 (EP1041y, Abcam), FAK (3286, CST), phosphorylated (p)-FAK Y397 (sc-11765-R, Santa Cruz), ISLET1 (09218-2E7, Abnova), GATA4 (sc-25310, Santa Cruz), lamin B (sc-6217, Santa Cruz), glyceraldehyde-3-phosphate dehydrogenase (GAPDH; ab-9484, Abcam), NICD1 (ab8925, Abcam), and HES1 (sc-13844, Santa Cruz).

Table 1
Primers used for quantitative real-time RT-PCR.

Primers	Sequence (5′–3′)	Product size
18s rRNA	F: CTGCTTTTCAGCAACTGGTCA R: TAGGACTGGCTACCATGCTGT	151 bp
Islet-1	F: CTGCTTTTCAGCAACTGGTCA R: TAGGACTGGCTACCATGCTGT	123 bp
Gata4	F:CACCCCAATCTCGATATGTTTGA R: GGTTGATGCCGTTCATCTTGT	151 bp
α-Mhc	F: GCCCAGTACCTCCCGAAAGTC R:CCATCCAATCGGTAGTAGCG	110 bp
β-Mhc	F: ACAACCCCTACGATTATGCGT R: ACGTCAAAGGCAGTATCCGTG	121 bp
Integrin α1	F: TACCGTGTCACCCTTGATTC R: TCCTGCAAGTCGTGCTTATC	129 bp
Integrin α3	F: GTGACTAATGCTTTCTGCTC R: TAGGTCTGACGAAAGATGTA	132 bp
Integrin α5	F: TGGTTCGGAGCAACAGTTCG R: CCAAAATCTGAGCGGCAAGG	120 bp
Integrin α7	F: GGGAGGTGCTGTGTATGTGT R: AAGACCTTCCCATCTCCGTC	123 bp
Integrin β1	F: ATAGAGAATCCCAGAGCTC R: GGTAATCTTCAGCCCTCTTG	120 bp
Integrin β3	F: AGAAATCCAAAGAGCAGAAG R: ATGAGGATGTAGTTCTTGGC	123 bp
SFRP1	F: CCAGCGAGTACGACTACG R: CCTGCTGCTTCACCTCTG	111 bp
SFRP2	F: CAGCCCGACTTCTCCTAC R: CTCCAGCACCTCCTTCAT	117 bp
SFRP3	F: TGGTAAACATTCCAAGGGAC R: GACTTTCTTACCAAGCCGAT	126 bp
SFRP4	F: ACAGCACTCAGGAGAACG R: AGGAACTCCAGGGTACAG	108 bp
SFRP5	F: GCCTCCAGTGACCAAGAT R: CAATCAACTTTCGGTCCC	108 bp
Hes-1 (for ChIP)	F: TCCTCCCATTGGCTGAA R: GGCCCTGGCGGCCTCTAT	121 bp

2.5. Immunofluorescence staining

Immunofluorescence staining was performed as previously described [15]. Nuclei were counterstained with Hoechst 33342 (Sigma-Aldrich). A minimum of five randomly imaged fields from each coverslip were counted from at least five coverslips. P19CL6 cells were incubated with antibodies against p-FAK Y397 (1:200; sc-11765-R, Santa Cruz) and against NICD1 (1:100; ab8925, Abcam) and subsequently incubated with tetramethylrhodamine isothiocyanate (TRITC)-conjugated goat anti-rabbit immunoglobulin G (IgG, 1:200; Santa Cruz) or fluorescein isothiocyanate (FITC)-conjugated rabbit anti-goat IgG (1:200; Santa Cruz) for 30 min at room temperature. Immunofluorescence staining was visualized under an Olympus FV1000 confocal laser scanning microscope (Tokyo, Japan).

2.6. Luciferase assay

The luciferase (Luc) assay was performed as previously described [15]. The plasmid carrying the HES1-Luc reporter gene was a kind gift from Prof. Diane S. Krause (University of Yale). The pGL-Basic plasmid was co-transfected as the internal control. Luciferase activity was measured and normalized to *Renilla* luciferase activity. All experiments were performed in triplicate.

2.7. Coimmunoprecipitation (CoIP) assay

CoIP was performed according to the manufacturer's instructions (Roche). P19CL6 cell lysates were incubated with antibodies against

ITGα1 (sc-271034, Santa Cruz) and ITGβ1 (EP1041y, Abcam) or normal IgG for 2 h with gentle rotation, and then with 50 μl protein G Sepharose slurry (Roche) at 4 °C for 1 h. Immunoprecipitates were washed three times with wash buffer, subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE), and then detected with anti-SFRP4 antibody.

2.8. Chromatin immunoprecipitation (ChIP) assay

ChIP was performed according to a previously described method [14]. The antibodies used for immunoprecipitation were anti–p-FAK Y397 (sc-11765-R, Santa Cruz) and anti-NICD1 (sc-28713, Santa Cruz) antibodies. The primers used for the *Hes1* promoter were 5′-TCCTCC CATTGGCTGAAA-3′ (forward) and 5′-GGCCCTGGCGGCCTCTAT-3′ (reverse). The target product length was 101 bp.

2.9. Statistical analysis

The data are reported as the mean ± standard deviation (SD). Comparisons between groups were analyzed by one-way analysis of variance (ANOVA). Significance was analyzed with SPSS 10.0 software and $P < 0.05$ was considered statistically significant.

3. Results

3.1. SFRP4 expression is stage-specific during P19CL6 cell cardiac differentiation

After 1% DMSO treatment, P19CL6 cells underwent cardiac differentiation as indicated by the upregulation of ISLET1, GATA4, α–myosin heavy chain (α-MHC), and β-MHC. These results confirmed that P19CL6 cells successfully differentiated into cardiomyocytes (Fig. 1A and B).

Next, we examined the SFRP family expression profile (Fig. 1C). SFRP2, SFRP3, and SFPR5 were nearly undetectable; SFRP1 was expressed throughout the differentiation. Interestingly, SFRP4 expression showed a stage-specific pattern (Fig. 1C and D). SFRP4 was upregulated earlier and peaked at day 6, indicating that it may participate in the cardiac differentiation of P19CL6 cells.

3.2. SFRP4 inhibits cardiac gene expression in P19CL6 cells

The expression of cardiac genes was decreased in cultures to which 100 ng/ml SFRP4 recombinant proteins had been added during induction as compared to the control (Fig. 2A and B), demonstrating the role of SFRP4 in P19CL6 cell differentiation. At the same time, transfecting 100 nM *Sfrp4*-specific siRNA (si-*Sfrp4*) enhanced the expression of the genes (Fig. 2C and D). These results indicate that SFRP4 inhibits the expression of cardiac genes during P19CL6 cell differentiation.

3.3. SFRP4 overexpression upregulates Notch1 and HES1

We showed that SFRP4 inhibits the cardiac differentiation of P19CL6 cells. However, the detailed mechanism of SFRP4 remains unclear. Wnt and Notch1 can co-function to regulate cardiac differentiation; consequently, we attempted to determine whether SFRP4, a member of the Wnt family, can regulate cardiac differentiation via cross-talk between the Wnt and Notch1 pathways.

We examined the expression profiles of Wnt and Notch pathway molecules (Fig. S1). Wnt3a was expressed at day 3, while that of AXIN2 and β-catenin were expressed after day 4. Notch1 expression increased at day 2, and then declined. HES1 increased and peaked at day 6, and then decreased, which was similar to SFRP4 expression.

Next, we assessed the effects of SFRP4 on cross-talk between the Wnt and Notch1 signaling pathways. As shown in Fig. 3A and B, compared to the vector control at day 12 following DMSO induction,

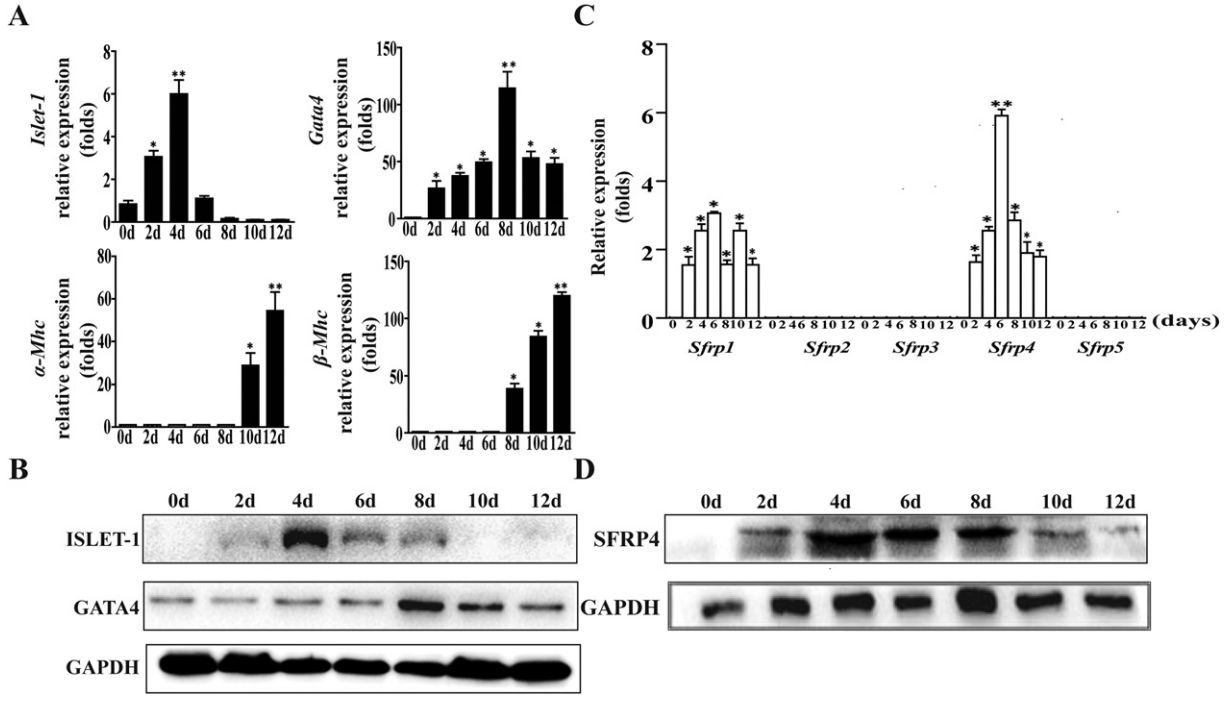

Fig. 1. The expression profiles of cardiac-specific genes and SFRP family members during P19CL6 cell cardiac differentiation. (A, B) Real-time RT-PCR and western blot detection of *Islet1*, *Gata4*, α-*Mhc*, and β-*MHC* expression in DMSO-induced P19CL6 cells. (C) Real-time RT-PCR examination of SFRP family member (*Sfrp1*, *Sfrp2*, *Sfrp3*, *Sfrp4*, *Sfrp5*) expression. (D) Western blotting examination of the SFRP4 expression profile. Real-time RT-PCR data are from three independent experiments, each performed in triplicate. Bars represent the mean ± SD from three samples (*$P < 0.05$, **$P < 0.01$ vs. 0 days). Western blotting images are representative of three independent experiments; GAPDH was used as the internal control.

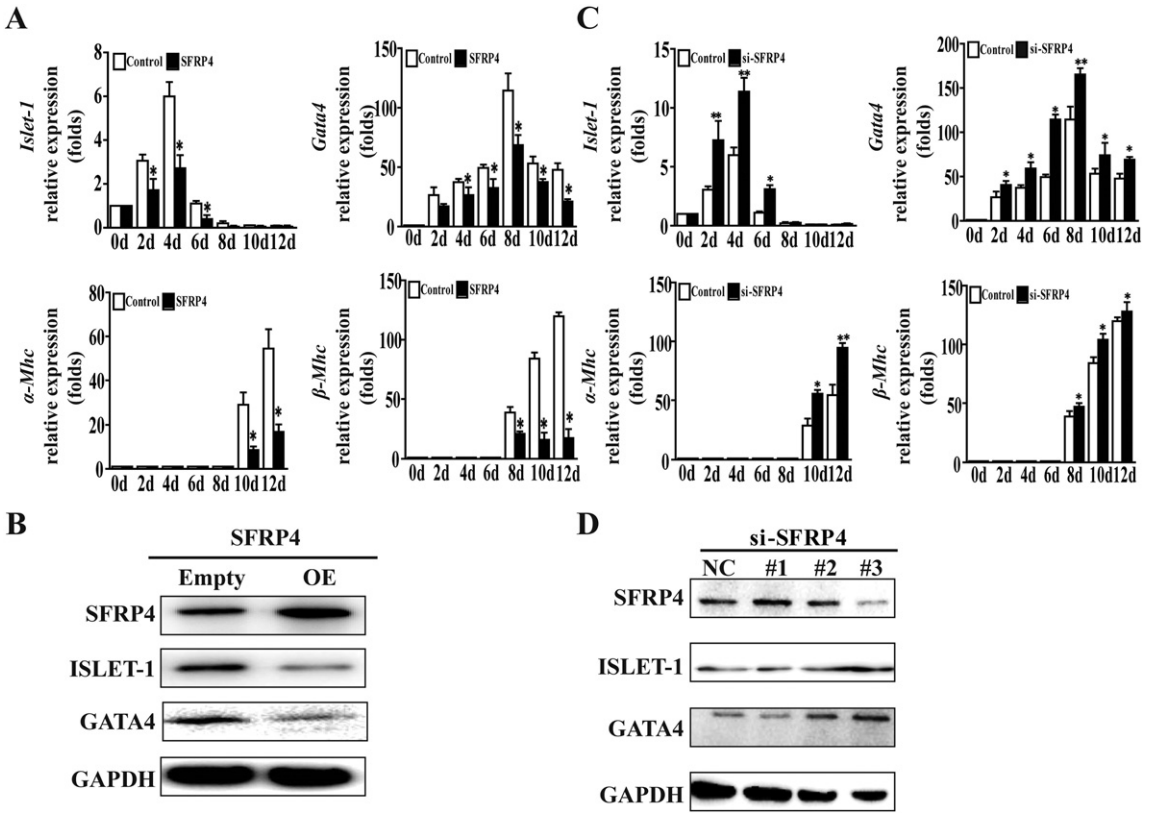

Fig. 2. SFRP4 inhibits the expression of cardiac-specific genes during P19CL6 cell cardiac differentiation. (A, B) Real-time RT-PCR and western blotting analysis of *Islet1*, *Gata4*, α-*Mhc*, and β-*Mhc* expression in P19CL6 cells treated with recombinant SFRP4 protein (100 ng/ml) during DMSO induction (OE, SFRP4 overexpression). Cells without SFRP4 recombinant proteins (Empty) were used as the control. (C, D) Real-time RT-PCR and western blot analysis of ISLET1, GATA4, α-MHC, and β-MHC expression in P19CL6 cells transfected with si-*Sfrp4*. Cells treated with non-silencing RNA were used as the control. Real-time RT-PCR data are from three independent experiments, each performed in triplicate. Bars represent the mean ± SD from three samples (*$P < 0.05$, **$P < 0.01$ vs. control of the same day). Western blotting images are representative of three independent experiments; GAPDH was used as the internal control.

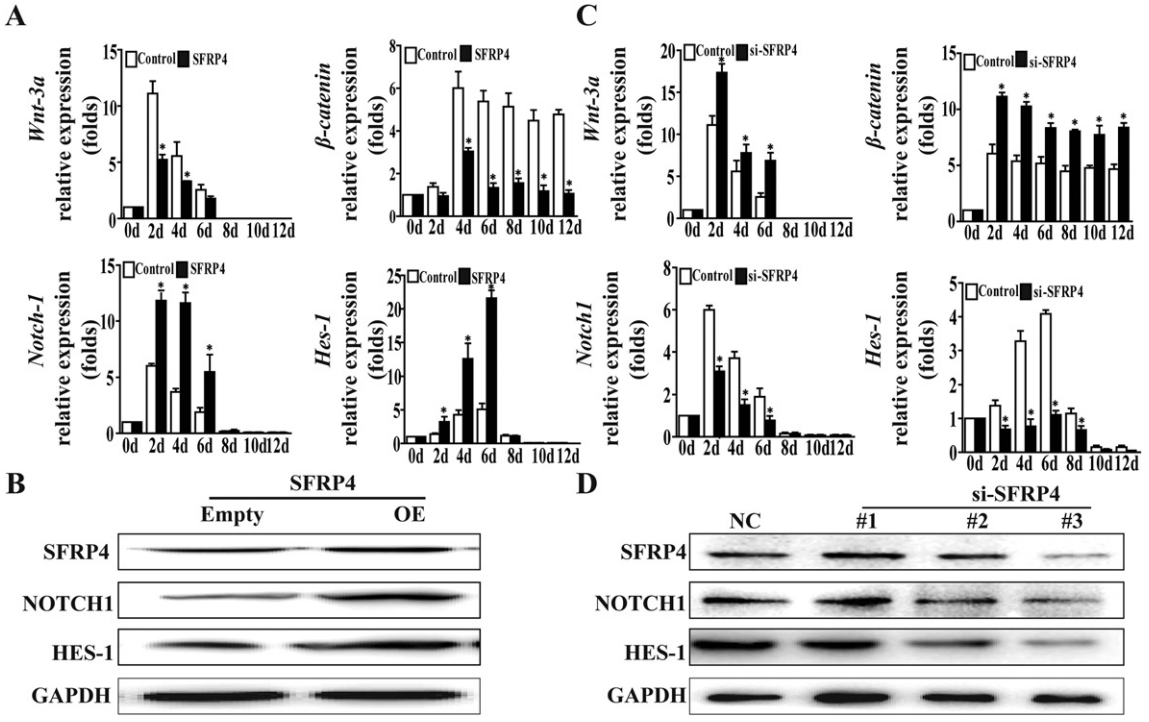

Fig. 3. SFRP4 overexpression upregulates the Notch1 signaling pathway. (A, B) Real-time RT-PCR and western blot detection of Wnt3a, β-catenin, Notch1, and HES1 expression in P19CL6 cells after SFRP4 treatment (OE, SFRP4 construct transfection). Cells treated with pcDNA3.1 were used the control (Empty). (C, D) Real-time RT-PCR and western blot detection of Wnt3a, β-catenin, Notch1, and HES1 expression in P19CL6 cells treated with si-*Sfrp4*. Cells treated with non-silencing RNA were used as the control. Real-time RT-PCR data are from three independent experiments, each performed in triplicate. Bars represent the mean ± SD from three samples (*$P < 0.05$ vs. control of the same day). Western blotting images are representative of three independent experiments; GAPDH was used as the internal control.

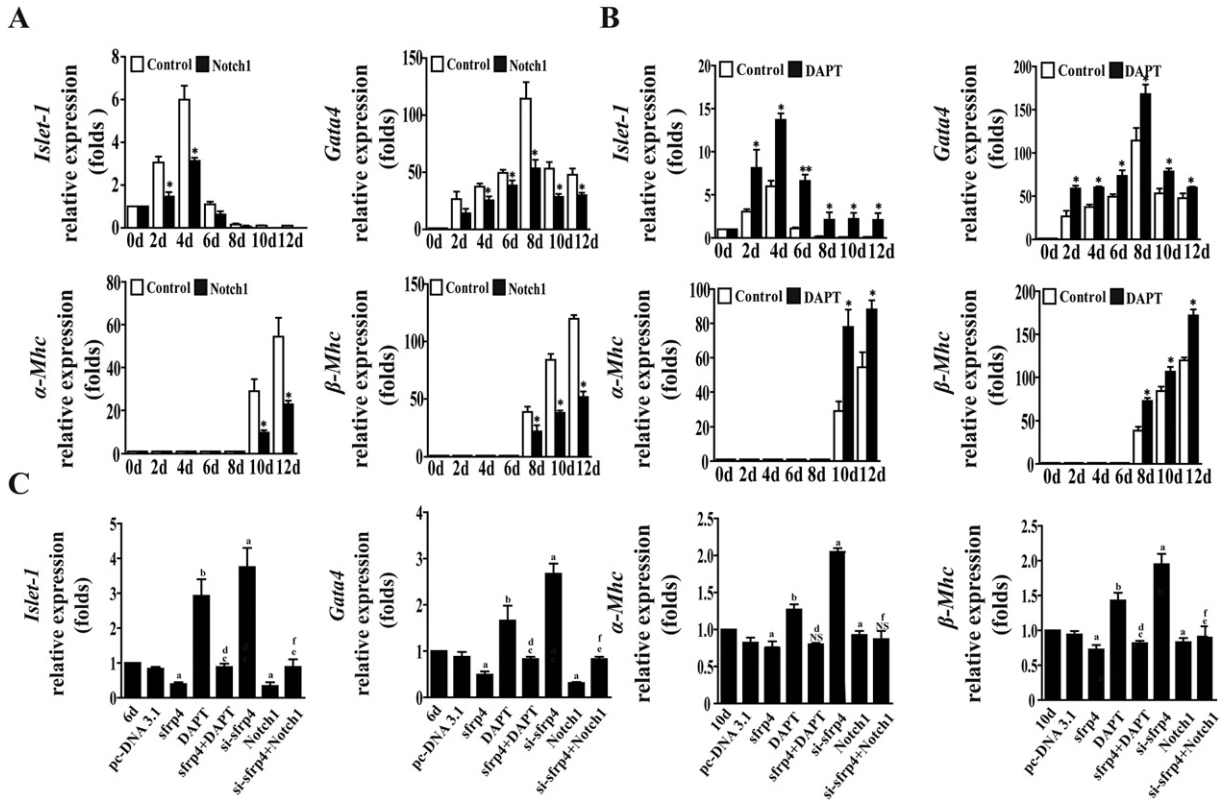

Fig. 4. The SFRP4–Notch1 signaling pathway inhibits P19CL6 cell cardiac differentiation. (A) Real-time RT-PCR detection of *Islet1*, *Gata4*, α-*Mhc*, and β-*Mhc* expression in P19CL6 cells treated with Notch1. Cells treated with pcDNA3.1 were used as the control. (B) Real-time RT-PCR detection of *Islet1*, *Gata4*, α-*Mhc*, and β-*Mhc* expression in DAPT-treated P19CL6 cells. Cells untreated with DAPT were used as the control. (C) Real-time RT-PCR analysis of *ISLET1*, *GATA4*, α-*MHC*, and β-*MHC* expression in P19CL6 cells treated with pcDNA3.1, SFRP4, DAPT, SFRP4 + DAPT, si-*Sfrp4*, Notch1, or si-*SFRP4* + Notch1. 18S rRNA served as the internal control. Data are the mean ± SD (n = 3). $^{*}P < 0.05$, $^{**}P < 0.01$ vs. 0 days; $^{a}P < 0.05$ vs. pcDNA-3.1; $^{b}P < 0.05$ vs. P19CL6 (6 days); $^{c}P < 0.05$ vs. SFRP4; $^{d}P < 0.05$ vs. DAPT; $^{e}P < 0.05$ vs. Notch1; $^{f}P < 0.05$ vs. si-*Sfrp4*. Western blotting images are representative of three independent experiments; GAPDH was used as the internal control.

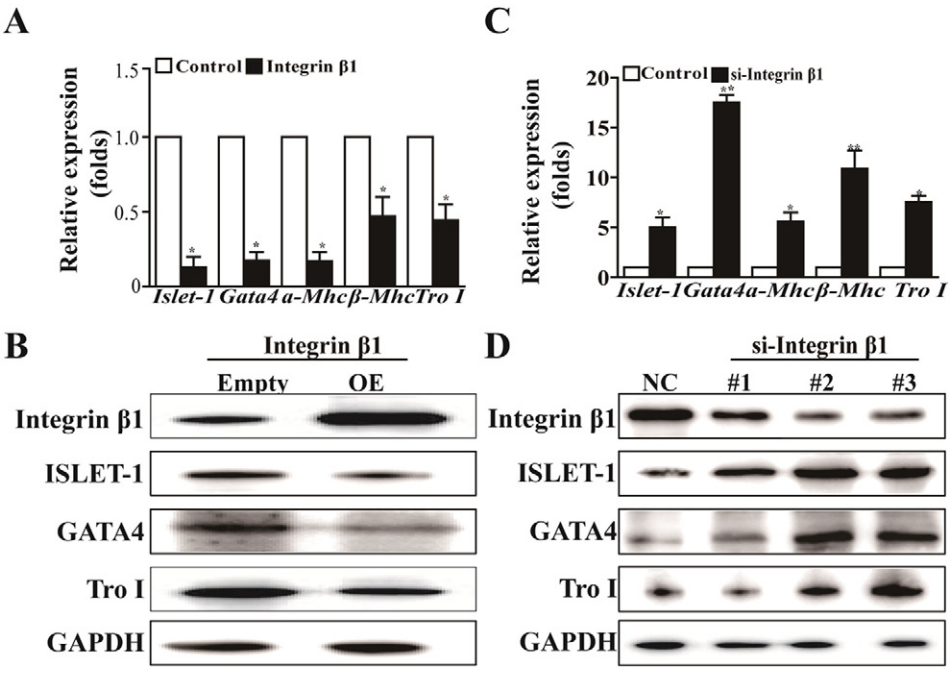

Fig. 5. ITGα1β1 inhibits P19CL6 cell cardiac differentiation. (A, B) Real-time RT-PCR and western blotting detection of ISLET1, GATA4, α-MHC, β-MHC, and TNNI3 expression in P19CL6 cells treated with ITGβ1 (OE, ITGβ1 overexpression). Cells treated with pcDNA3.1 were used as the control (Empty). (C, D) Real-time RT-PCR and western blotting detection of ISLET1, GATA4, α-MHC, β-MHC, and TNNI3 expression in P19CL6 cells treated with ITGβ1 siRNA. Cells treated with non-silencing RNA were used as the control. Real-time RT-PCR data are from three independent experiments, each performed in triplicate. Bars represent the mean ± SD from three samples ($^{*}P < 0.05$, $^{**}P < 0.01$ vs. control of the same day). Western blotting images are representative of three independent experiments; GAPDH was used as the internal control.

SFRP4 overexpression decreased Wnt3a, β-catenin, and AXIN2 (Fig. S2) expression, but enhanced Notch1 and HES1 expression. At the same time, transfection with 100 nM si-*Sfrp4* increased Wnt3a and β-catenin production, but decreased Notch1 and HES1 levels (Fig. 3C and D, Fig. S2). Therefore, we investigated the effects of the Notch1 signaling pathway on cardiac differentiation. Real-time RT-PCR demonstrated that cardiac genes were downregulated when P19CL6 cells were transfected with Notch1 (Fig. 4A). We also treated P19CL6 cells with the Notch1 signaling pathway inhibitor DAPT (100 ng/ml) for 12 days. Notch1 knockdown increased cardiac gene expression (Fig. 4B). These results are indicative of the effect of Notch1 inhibition on P19CL6 cell cardiac differentiation.

Rescue assay was performed to examine the effect of SFRP4 and Notch1 signaling molecule cross-talk on of P19CL6 cell cardiac differentiation (Fig. 4C). The expression levels of the cardiac-specific genes *Islet1*, *Gata4*, α-*Mhc*, and β-*Mhc* were rescued by SFRP4 overexpression, DAPT, si-*SFRP4*, Notch1, or combinations thereof. These results indicate that SFRP4 and Notch signaling might interact to influence cardiomyocyte differentiation. Accordingly, we used CoIP to examine the possible interactions between them. However, there was no direct interaction between SFRP4 and Notch1 (data not shown).

3.4. SFRP4–ITGα1β1 activates FAK phosphorylation and inhibits cardiac differentiation

The SFRP4 CRD interacts with other membrane receptors in addition to Wnt ligands or receptors. SFRP1 inhibited ITGα3β1 in MDA-MB-231 breast carcinoma cells [16]. We hold the view that SFRP4 interacts with integrin receptors during P19CL6 cell differentiation.

We detected the expression profile of integrin molecules present in the cardiomyocytes (Fig. S3). The similar expression patterns of ITGα1, ITGβ1, and SFRP4 indicated that the molecules are correlated.

To clarify the effect of ITGα1β1 on cardiac differentiation, we transfected pcDNA3.1-Integrin β1 or ITGβ1 siRNA (si-integrin β1) into P19CL6 cells. *Islet1*, *Gata4*, α-*Mhc*, β-*Mhc*, and troponin I (*Tnni3*) levels were decreased after ITGβ1 overexpression (Fig. 5A and C); while ITGβ1 knockdown increased these markers (Fig. 5B and D), suggesting that ITGα1β1 influences P19CL6 cell cardiac differentiation.

To explore the correlation of SFRP4 and ITGα1β1, we overexpressed or knocked down SFRP4 in P19CL6 cells. SFRP4 promoted the expression of ITGβ1 and ITGα1 (Fig. 6A).

We performed a CoIP assay to test the interaction between SFRP4 and ITGα1β1. SFRP4 interacted with ITGα1β1 on the plasma membrane, which indicated that ITGα1β1 could be a new interaction pathway with SFRP4 (Fig. 6B).

We established that activation of the integrin signaling pathway led to FAK Y397 autophosphorylation. FAK Y397 expression was enhanced at day 6 compared to that on day 0 (Fig. 7A). SFRP4 overexpression promoted FAK Y397 expression as compared to the controls (Fig. 7B). Conversely, SFRP4 knockdown decreased p-FAK Y397 levels (Fig. 7C). These results indicate that SFRP4 promotes FAK Y397 phosphorylation.

The co-functioning of SFRP4 and ITGα1β1 on P19CL6 cell cardiac differentiation was explored in a rescue assay. The expression of *Islet1* and other cardiac genes was restored by SFRP4 overexpression and FAK inhibitor or si-*Sfrp4* and FAK (Fig. 7D).

Collectively, these results indicate that SFRP4 and ITGα1β1 can form a complex to inhibit P19CL6 cell cardiac differentiation through the activated FAK pathway. ITGα1β1 signaling may serve as a non–Wnt-dependent pathway that interacts with SFRP4.

3.5. Nuclear FAK and NICD1 form a complex and inhibit P19CL6 cell cardiac differentiation

We proved that SFRP4 can combine with ITGα1β1 and activate the ITGα1β1–FAK signaling pathway. However, the interaction between SFRP4 and the Notch1 pathway remains unknown.

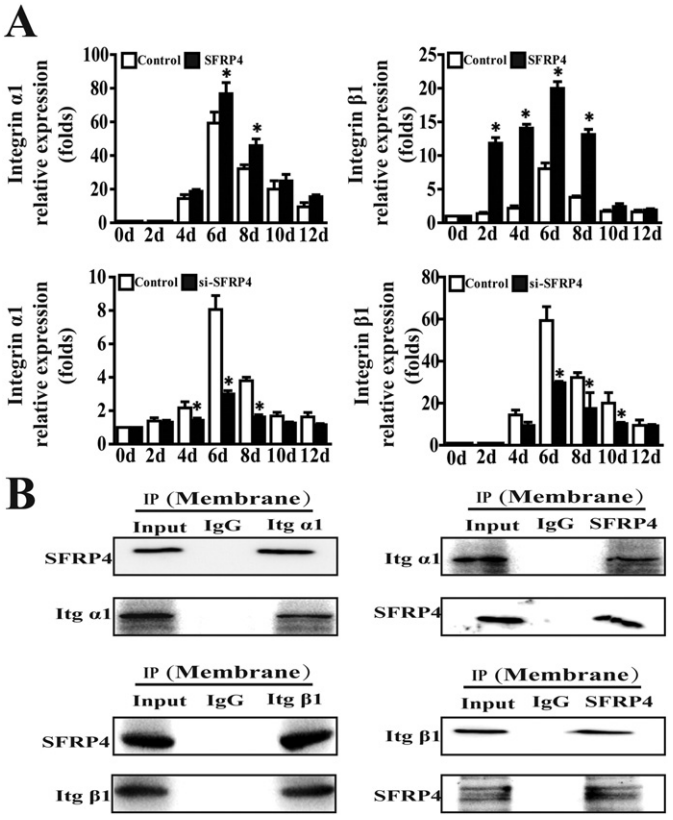

Fig. 6. SFRP4 interacts with ITGα1β1. (A, top) Real-time RT-PCR detection of ITGα1 and ITGβ1 expression in P19CL6 cells treated with SFRP4. Cells treated with pcDNA 3.1 were used as the control. (A, bottom) Real-time RT-PCR detection of ITGα1 and ITGβ1 expression in P19CL6 cells treated with si-*Sfrp4*. Cells treated with non-silencing RNA were used as the control. (B) CoIP detection of the ITGα1β1–SFRP4 complex in the membrane of P19CL6 cells. Real-time RT-PCR data are from three independent experiments, each performed in triplicate. Bars represent mean ± SD from three samples (*$P < 0.05$ vs. control of the same day). Western blotting images are representative of three independent experiments.

We examined the co-function of SFRP4 and FAK on Notch1 and HES1 expression. Notch1 and HES1 production was rescued by SFRP4 overexpression and FAK inhibitor, or si-*Sfrp4* and FAK, respectively. The results provide an indication that SFRP4 and FAK co-function to promote Notch1 and HES1 expression (Fig. 8A). FAK overexpression enhanced NICD1 nuclear translocation (Fig. 8B).

The immunofluorescence assay demonstrated that NICD1 and p-FAK Y397 colocalize in the nuclei (Fig. 8C); CoIP showed that p-FAK Y397 and NICD exist a complex (Fig. 8D).

Phosphorylated FAK Y397 could act as a transcriptional regulator; herein, HES1–Luc activity was increased most significantly following FAK and NICD1 plasmid transfection (Fig. 9A). ChIP showed that NICD1 and FAK were recruited to the *Hes1* promoter region (Fig. 9B). Accordingly, FAK and NICD1 are present in a complex to activate *HES1* transcription effectively.

FAK and NICD1 co-function in P19CL6 cell cardiac differentiation was investigated using rescue assay (Fig. 9C). The expression levels of the cardiac genes *Islet1*, *Gata4*, α-*Mhc*, and β-*Mhc* were rescued by DAPT and FAK overexpression or FAK inhibitor and Notch1 overexpression. These data demonstrate that the FAK–NICD1 complex decreases the expression levels of these cardiac genes by recruitment to the promoter of *Hes1*, the downstream gene of Notch1, to activate its transcriptional activity.

4. Discussion

Heart diseases remain a major worldwide healthcare burden. Recent advances in stem cell biology have made it feasible to replace

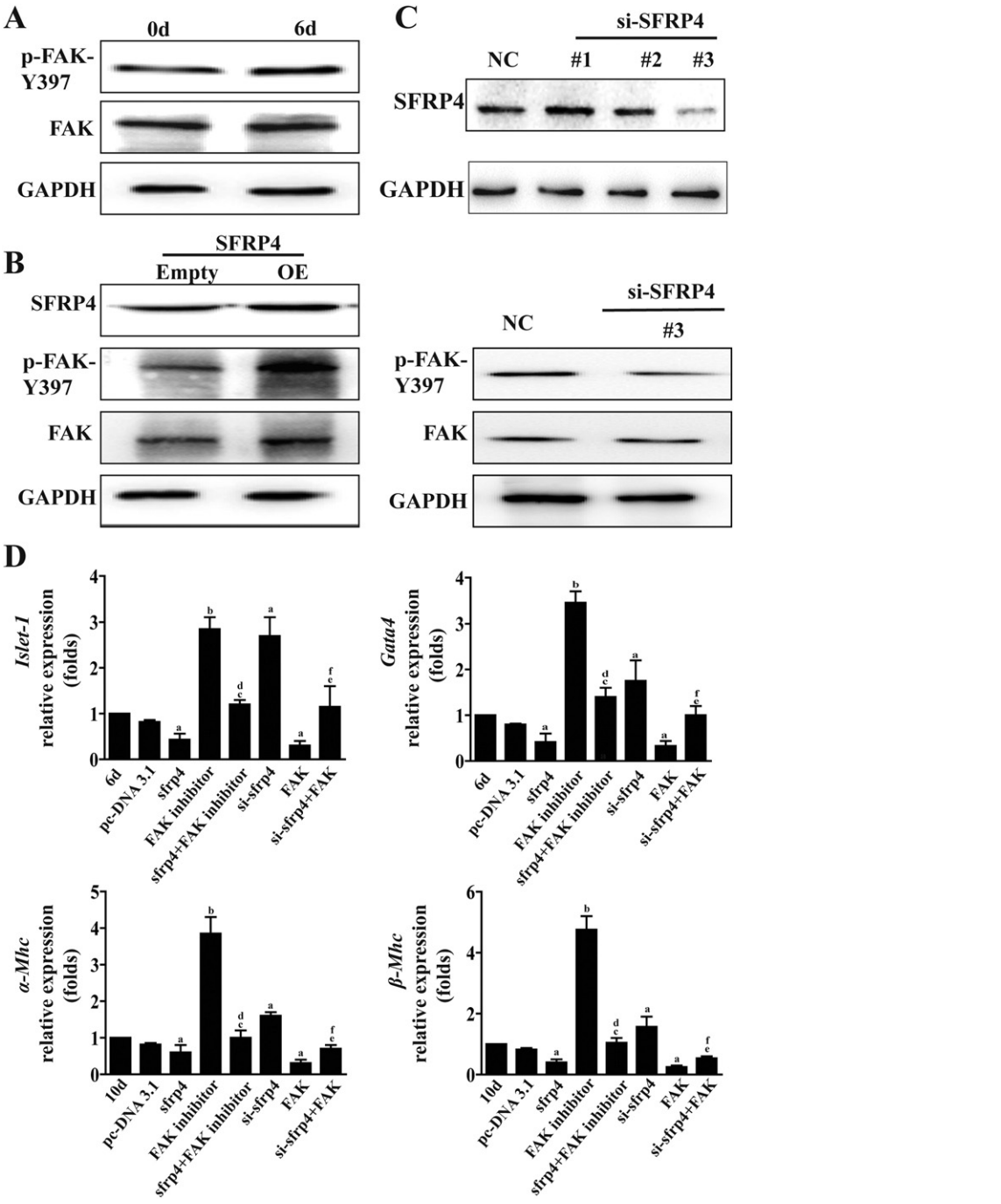

Fig. 7. SFRP4 activates the ITGα1β1 signaling pathway. (A) Western blot analysis of p-FAK Y397 and FAK expression on day 0 and day 6 in P19CL6 cells. (B) Western blot detection of p-FAK Y397 and FAK expression in P19CL6 cells with SFRP4 overexpression (OE). Cells treated with pcDNA 3.1 (Empty) were used as the control. (C) Western blot detection of p-FAK Y397 and FAK expression in P19CL6 cells treated with si-*SFRP4*. Cells treated with non-silencing RNA were used as the negative control (NC). (D) Real-time RT-PCR analysis of *Islet1*, *Gata4*, α-*Mhc*, and β-*Mhc* expression following treatment with SFRP4, FAK inhibitor, si-*Sfrp4*, FAK, SFRP4 + FAK inhibitor, or si-*Sfrp4* + FAK. Cells treated with pcDNA 3.1 were used as the control. 18S rRNA served as the internal control. The data are from three independent experiments. Bars represent the mean ± SD from three samples ($^{*}P < 0.05$, $^{**}P < 0.01$ vs. 0 days; $^{a}P < 0.05$ vs. pcDNA-3.1; $^{b}P < 0.05$ vs. P19CL6 (6 days); $^{c}P < 0.05$ vs. SFRP4; $^{d}P < 0.05$ vs. FAK inhibitor; $^{f}P < 0.05$, $^{e}P < 0.05$ vs. FAK vs. si-*Sfrp4*). Western blotting images are representative of three independent experiments.

damaged cardiomyocytes [17]. However, the detailed mechanisms of cardiomyocyte differentiation remain unclear, which restrict the obtainment of myocytes from stem cells.

In the present study, we explored the effect of SFRP4 on cardiac differentiation. SFRP4 accelerates adipogenic differentiation in human adipose tissue–derived mesenchymal stem cells [18], and it can sensitize glioma stem cells (GSCs) to chemotherapeutics [19]. However, there has been no report on SFRP4 in cardiac differentiation. We first determined that SFRP4 can inhibit cardiac differentiation in P19CL6 cells. It is notable that SFRP1 is expressed throughout P19CL6 cell differentiation into cardiomyocytes. SFRP1 is strongly induced in differentiating heart muscle and is required for the formation of normal-sized heart muscle in the embryo [20]. In addition, the loss of SFRP1 leads to abnormal cardiac function in the form of aberrant expression of Wnt ligands (Wnt1, 3, 7b, and 16) and Wnt target genes (*Wisp1* and *Lef1*) in aged hearts [16].

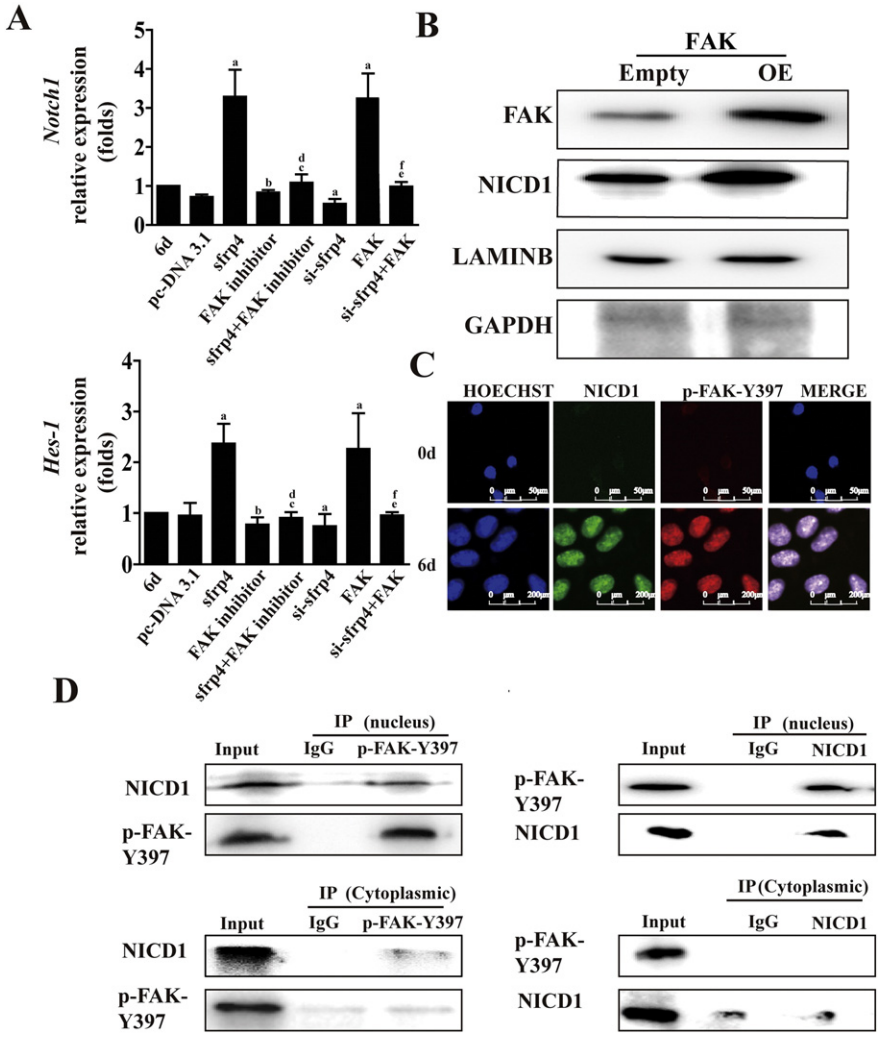

Fig. 8. ITGα1β1 promotes the Notch1 signaling pathway. (A) Real-time RT-PCR detection of Notch1 and HES1 expression in P19CL6 cells treated with SFRP4, FAK inhibitor, si-*Sfrp4*, FAK, SFRP4 + FAK inhibitor, or si-*Sfrp4* + FAK. Cells treated with pcDNA3.1 were used as the control. (B) Western blot detection of FAK and NICD1 expression in P19CL6 cells on day 0 and day 6 after FAK construct transfection (OE) or pcDNA3.1 transfection (Empty). The data are from three independent experiments. Bars represent the mean ± SD from three samples ($^{*}P < 0.05$, vs. pGL-Basic; $^{a}P < 0.05$ vs. pcDNA-3.1; $^{b}P < 0.05$ vs. P19CL6 (6 days); $^{c}P \le 0.05$ vs. SFRP4; $^{d}P \le 0.05$ vs. FAK inhibitor; $^{e}P \le 0.05$ vs. FAK; $^{f}P \le 0.05$ vs. si-*Sfrp4*). (C) NICD1 and FAK Y397 expression in P19CL6 cells at day 0 and day 6 was analyzed by immunofluorescence staining with monoclonal antibody against NICD1 (green) and p-FAK Y397 (red). Nuclei were counterstained with Hoechst 33342 (blue). (D) CoIP was performed in P19CL6 cells to detect the NICD–FAK Y397 complex in the nuclear and cytoplasmic proteins.

SFRP family members are generally considered Wnt antagonists. They compete for Wnt ligands because they share the same CRD with the Wnt receptor. The SFRP4 CRD also can interact with other receptors, which indicates that SFRP4 may play its role through a non–Wnt-dependent signaling pathway [5]. We found that SFRP4 interacted with ITGα1β1. However, SFRP4 may also bind to other integrin molecules, which requires further confirmation. There are three main pathways downstream of the integrin pathway, namely the integrin-linked kinase (ILK), FAK, and SRC pathways, of which the FAK pathway is a major pathway. FAK Y397 is autophosphorylated when the integrin signaling pathway is activated. During human ESC (hESC) differentiation, ITGα6 levels diminish and FAK Y397 is phosphorylated and activated [21]. Administration of SFRP1 recombinant protein to the testis in vivo delayed spermiation, which was accompanied by the downregulation of p-FAK Tyr397 [22]. In our study, upregulated SFRP4 leaded to p-FAK Y397 upregulation, implying that SFRP4 can activate the ITGα1β1–FAK signaling pathway.

FAK is a non-receptor tyrosine kinase bearing a central kinase domain flanked by an N-terminal band 4.1, ezrin, radixin, moesin homology (FERM) domain and a C-terminal region containing a focal adhesion targeting (FAT) domain and several proline-rich motifs [23]. We demonstrated that FAK can interact with NICD1 to activate the promoter activities of target genes. The Notch signaling pathway plays a critical role during mammalian cardiac development. Gain- and loss-of-function experiments for Notch signaling components have shown that this pathway is a crucial regulator of cardiomyocyte differentiation in ESCs [9]. Notch1 inactivation favors ESC differentiation into cardiomyocytes, whereas endogenous Notch signaling promotes ESC differentiation into the neuronal lineage [8]. Our data reveal that Notch1 inhibits the cardiomyocyte differentiation of P19CL6 cells by interacting with FAK and NICD1.

5. Conclusions

Our results provide evidence for cross-talk between SFRP4, ITGα1β1, and Notch1 for inhibiting P19CL6 cell cardiac differentiation. SFRP4 binds with ITGα1β1 to activate the FAK pathway. FAK Y397 is autophosphorylated and enters the nucleus, where it interacts with NICD1 to promote the transcriptional activities of the target cardiac genes to decrease their expression. Consequently, SFRP4 inhibits cardiac differentiation via cross-talk of the ITGα1β1 and Notch1 pathways.

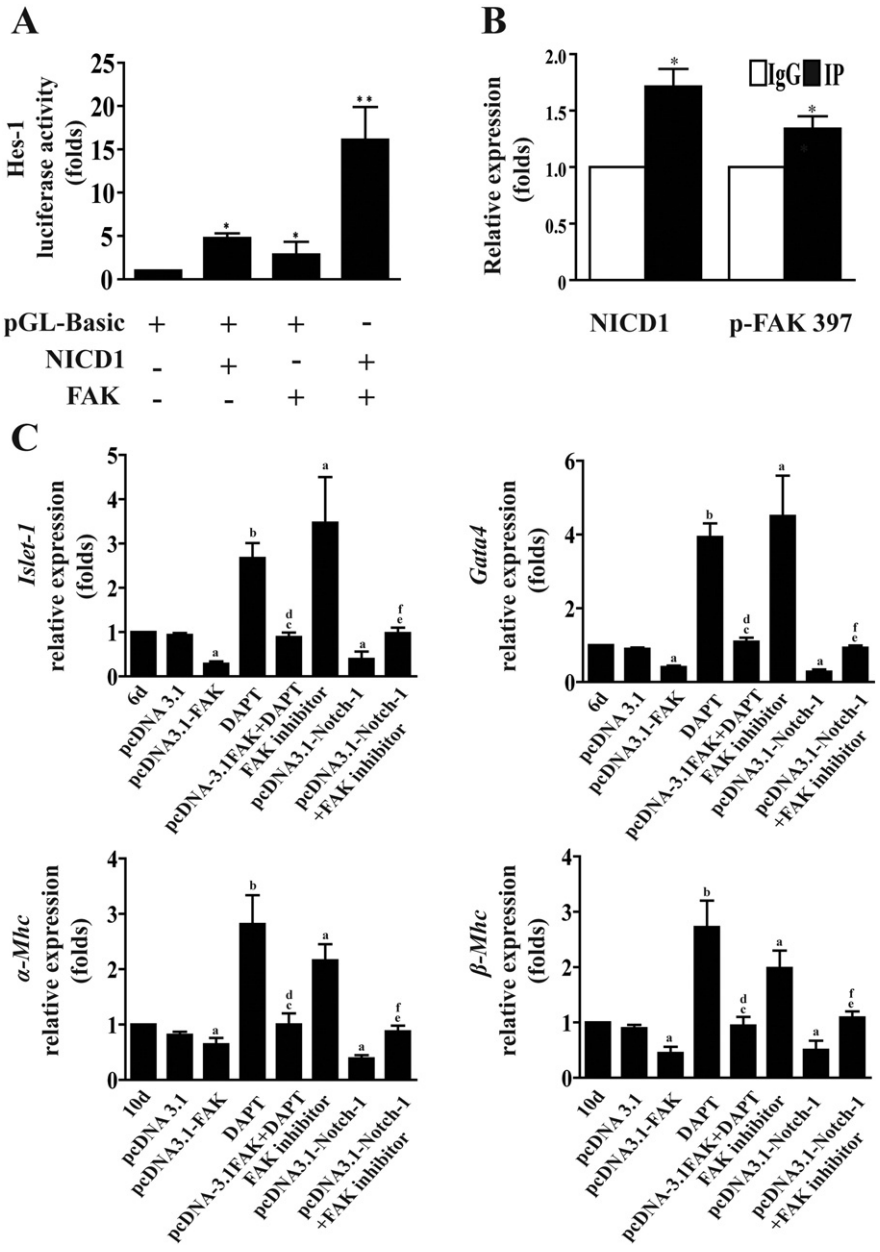

Fig. 9. FAK and NICD1 form a complex to inhibit P19CL6 cell cardiac differentiation. (A) HES1 luciferase activity was measured at day 6 after transfection with FAK, NICD1, or FAK + NICD1. Cells treated with pGL-Basic were used as the control. (B) ChIP analysis of the NICD1–FAK Y397 complex recruited to the HES1 promoter. Soluble chromatin was prepared from P19CL6 cells at day 6 followed by immunoprecipitation with antibodies against NICD1 or FAK Y397. The extracted DNA was amplified by real-time RT-PCR using primers that covered the NICD-binding sites on the *Hes1* enhancer region. (C) Real-time RT-PCR analysis of P19CL6 cells treated with FAK, DAPT, FAK inhibitor, Notch1, FAK + DAPT, or Notch1 + FAK inhibitor. Cells treated with pcDNA3.1 were used as the control. The data are from three independent experiments. Bars represent the mean ± SD from three samples ($^{*}P < 0.05$, vs. pGL-Basic; $^{a}P < 0.05$ vs. pcDNA-3.1; $^{b}P < 0.05$ vs. P19CL6 (6 days); $^{c}P < 0.05$ vs. FAK; $^{d}P < 0.05$ vs. DAPT; $^{e}P < 0.05$ vs. Notch1; $^{f}P < 0.05$ vs. FAK inhibitor).

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.cellsig.2016.08.010.

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Declaration

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Competing interests

The authors declare no conflicts of interest.

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